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Mastication effects on carotenoid bioaccessibility from mango fruit tissue

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Abstract

The release of carotenoids from fresh fruits or vegetables is determined by the encapsulating plant tissue matrix, intracellular carotenoid location within the cell, and the mastication process. The objectives of this study were to assess the particle sizes obtained after mastication of mango fruit tissue, and how the resulting degree of plant tissue rupture affects carotenoid bioaccessibility. A fine and a coarse chewer were selected after screening 20 healthy volunteers for *in vivo* human mastication, and the collected chewed boluses were subjected to wet sieving fractionation, followed by an *in vitro* gastric and small intestinal digestion model. Confocal micrographs show that the smallest particle size fraction (0.075 mm) consists mostly of fragmented cells and the largest size fraction (2.8 mm) contains bulky clusters of whole cells and vascular fibres. Higher amounts of total carotenoids (211-320 µg/100g) were observed in the larger particle size fraction (2.8 mm) relative to the 1 mm (192-249 µg/100g) and 0.075 mm fractions (136-199 µg/100g). Smaller particles showed a greater % release of total carotenoids after *in vitro* digestion. Xanthophyll derivatives are more bioaccessible than β-carotene for all particle sizes. The effects of particle size or degree of fine vs coarse chewing are unexpectedly small ($p>0.05$) but the process of chewing substantially reduced the release of β-carotene and xanthophylls by 34% and 18% respectively. Whilst there is a (small) particle size effect, this appears to not be the primary factor controlling bioaccessibility for soft tissues such as mango, in contrast to previous reports that a single cell wall appears to be enough to prevent bioaccessibility of carotenoids in more robust carrot tissues.

Keywords: bioaccessibility; mango; carotenoids; digestion *in vitro*; chewing; plant cell wall

1. Introduction

Epidemiological studies have shown an inverse correlation between consumption of carotenoid-rich fruits and vegetables, and the incidence of cancers of the gastrointestinal tract (Kant, Block, Schatzkin, & Nestle, 1992; Kiokias & Gordon, 2004; Mayne, 1996; Rock & Swendseid, 1992), cardiovascular diseases (Krinsky, 1998; Murr et al., 2009), diabetes (Yeum & Russell, 2002), some inflammatory diseases (Perera & Yen, 2007), as well as age-related macular degeneration (Snodderly, 1995). The most documented function of β-carotene is its provitamin A activity, with consequent health benefits, such as maintenance of epithelial function, embryonic development, and immune system function (Diplock, 1991). Xanthophylls are only present in human retinal pigment epithelia, in contrast to other body sites where all other carotenoids occur (Bone, Landrum, Hime, Cains, & Zamor, 1993), and probably function as blue light filters and singlet oxygen quenchers (Seddon et al., 1994).

Human studies are most appropriate to predict nutrient bioavailability, but these studies have technical and ethical limitations (Netzel et al., 2011). Metabolic and physiological factors have been reported to influence the absorption, distribution and elimination of carotenoids (Bowen, Mobarhan, & Smith, 1993; Johnson, Qin, Krinsky, & Russell, 1997; Kostic, White, & Olson, 1995), resulting in inter-individual variability in plasma concentrations. In addition, host-related factors such as gut health, nutritional status or discrepancies, and genotype are typically encountered in most laboratory rodent models (Van Buggenhout et al., 2010). However, these factors can be avoided through the use of *in vitro* models. *In vitro* models are relatively easy to apply to large sample numbers, and are suitable for studying the effects of various digestion conditions or other factors linked to nutrient bioaccessibility (Fernandez-Garcia et al., 2012). *In vitro* digestion models can be used to simulate the physiological conditions of gastric and intestinal digestion. In addition, nutritional recommendations are often based on intakes or concentrations present in extracts of raw plant material, not taking into account bioaccessibility and any changes during gastrointestinal digestion. This could result in nutrient overestimation, and emphasises the importance of estimating bioaccessibility.

Current *in vitro* digestion procedures have proven useful for the analysis of carotenoid release and/or bioaccessibility (Castenmiller & West, 1998; Tydeman et al., 2010a). However, the reliability of the two-phase (stomach and small intestine) *in vitro* digestion model would be expected to be improved by including a ‘real’ chewing phase, or a phase that more closely mimics actual chewing behaviour and mechanics, which has been excluded in most digestion studies. Mastication is often the first step of food digestion, where the

process of breaking down solid foods into smaller particle sizes and mixing with saliva takes place. During simulated or real oral chewing, the physical barriers to the release of nutrients from plant cells may be ruptured. Therefore, the degree of cellular intactness could be indicative of their potential bioaccessibility, particularly as cell breakage is likely to be a major requirement for carotenoid bioaccessibility (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Tydeman et al., 2010b). Ideally, the structural properties of a food product digested *in vitro* should be similar to that of a chewed food bolus, since mastication varies subjectively between individuals, which impacts on food matrices and the structural properties of food boluses. Currently, simulated oral chewing has been mimicked using techniques such as pulverising, sieving, chopping or mincing (Woolnough, Monro, Brennan, & Bird, 2008), and the occasional inclusion of (salivary) α-amylase for starch digestion (Bornhorst, Hivert, & Singh, 2014; Miao et al., 2014). However, such mechanical steps do not adequately reflect the heterogeneous nature of chewed food. Epriliati, D'Arcy and Gidley (2009) demonstrated the importance of the simultaneous punch and gentle squash action of teeth, while Hoerudin (2012) found that mastication has a considerable effect on the cellular architectures of vegetables. In addition, mastication involves lubrication, softening and dilution with saliva (Lucas et al., 2006; Prinz & Lucas, 1995), and the formation of a cohesive bolus (Barry et al., 1995).

Mangoes are the second most important tropical fruit in terms of production and consumption, and have high carotenoid contents, particularly of β-carotene (Chen, Tai, & Chen, 2004; Yahia, Soto-Zamora, Brecht, & Gardea, 2007), which is responsible for the yellow-orange colour of ripe mango flesh (Pott, Breithaupt, & Carle, 2003). Current carotenoid studies have focused on the compositional profile or content (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010; Manthey & Perkins-Veazie, 2009; Mercadante & Rodriguez-Amaya, 1998; Robles-Sanchez et al., 2009), the impacts of ripening stages (Ornelas-Paz, Yahia, & Gardea, 2008), the presence of fat (Veda, Platel, & Srinivasan, 2007), and effects of processing (dried, fresh, juice) (Epriliati, D'Arcy, & Gidley, 2009). However, mastication effects on carotenoid gastrointestinal release from mango fruit have not been reported. Comparisons of the carotenoid content before and after *in vitro* digestion can provide information on their stability during gastrointestinal digestion. *In vitro* digestion models can be adapted to estimate the bioaccessibility of carotenoids by quantifying the fractions of phytonutrients transferred from the food matrix into the aqueous digesta or micellar phase, which then represents their potential for absorption or bioavailability. Studies have shown that the bioaccessibility of carotenoids can be as inefficient as 1.7% or as high as 100% (Tydeman et al., 2010a) depending on the type of carotenoids, as well as raw versus cooked conditions. The different solubility of polar xanthophylls and apolar carotenes can

also affect their ability to be incorporated into micelles and thus, affect both release and absorption efficiency.

It is hypothesised that the mechanism limiting carotenoid release involves intact cell walls (Tydeman et al., 2010a), which prevent the passage of carotenoids into lipid-soluble components or micelles, thus affecting bioaccessibility. The objective of this study was to investigate how the degree of mastication results in varying size distributions of ready to swallow bolus particles, and how this affects subsequent simulated gastrointestinal release of carotenoids from masticated mango tissue.

2. Materials and Methods

2.1. Plant material

Fully ripe mangoes (*cv. Kensington Pride*) were purchased from local stores in St. Lucia, Brisbane (Australia) 2-3 days before each of three chewing sessions, in the month of November 2012. Mango ripeness was selected based on typical eating maturity at stage 6 when the peel is yellow with pink-red blush and the flesh is slightly firm, according to the Department of Agriculture, Fisheries and Forestry (Queensland Government) mango-ripening guide (Primary Industries & Fisheries, 2012). Mangoes were stored at 4-6°C prior to the chewing sessions.

2.2. Chewing, blending, and bolus collection

Chewing experiments were approved by the Medical Research Ethics Committee at The University of Queensland (Ethical clearance No. 2012000683). Twenty healthy participants (aged 18-55) were recruited on the basis of frequent mango consumption and all gave informed consent to the study for mastication of fresh fruit. Individual mastication profiles from all the participants were studied for the selection of a fine and coarse chewer. Three independent chewing sessions were carried out on three different days of each consecutive week to account for inter-day variation. The chewing sessions were held between 9 and 11 am, after the chewers had consumed a light breakfast meal. 5-6 mangoes (300-600 g each) were cut into cubes and 300 g of cubes were randomly selected from the sample pile, and given to each of the fine and coarse chewer. The remaining cubes were combined and blended (Rocket blender DJL-1017, Cafe^{TM} Essentials, China) for 1 min to a puree to determine the carotenoid composition of the fresh mango. The chewers were instructed to chew the mango as per their habitual chewing behaviour, and to expectorate when they desired to swallow. The expectorated boluses were collected, washed with 70% ethanol to prevent further

biochemical changes, and fractionated via a wet sieving method, where water was flushed through a stack of sieves of apertures 5.6, 2.8, 1, 0.5 and 0.075 mm (Fig 1). The sieved particles were drained and collected for *in vitro* digestion. Chewing, fractionation, *in vitro* digestion and blending processes were carried out in a single day.

2.3. *In vitro* gastrointestinal digestion

Gastrointestinal conditions were modified from Hoerudin (2012). Gastric digestion (1 h) of puree and bolus samples (2±0.05 g) was initiated with 10 mL of emptying gastric secretion (130 mM NaCl, 5 mM KCl, 5 mM PIPES), followed by addition of 1 M HCl to reduce the pH to 2, and 1 mL porcine pepsin (1:2500 U/mg protein, Sigma-Aldrich, NSW, Australia) solution. Subsequently, transition from gastric to small intestinal phase was reflected by raising the pH to 6 with 1 M $NaHCO_3$. Small intestinal digestion (1 h) was mimicked by adding 5 mL pancreatin (lipase activity ≥8 USP U/mg, protease and amylase ≥4 USP U/mg, Chem Supply, Adelaide, Australia)-bile (Sigma-Aldrich, NSW Australia) extract, adjusting the overall pH to 7, and diluting with 5 mL intestinal salt secretion (120 mM NaCl, 5 mM KCl). To simulate physiological movement, the mixtures were incubated in a shaking water bath at 37°C, 55 rpm. Digesta samples were then centrifuged at 3000 g, 10 min (Centrifuge 5702R, Eppendorf, USA) to separate the bioaccessible fraction from residual pellet, flushed with nitrogen and stored at -80°C.

2.4. Carotenoid extraction

Carotenoid extractions of the puree, digesta and residual pellets were carried out the very next day after chewing and digestion, as modified from Ornelas-Paz et al., (2008). Puree (0.8 g) and digested pellets were vortex mixed with 2.5 mL and 1.5 mL PBS respectively. Digesta supernatants were homogenized three times with an Ultra-Turrax® at 4200 rpm with 20 mL petroleum ether:acetone (2:1) containing 0.1% BHT, or until the digesta pellets turned white. In between each homogenization step, samples were centrifuged at 3000 g for 5 min. Organic fractions were collected, combined, evaporated under nitrogen, dissolved in methanol:tetrahydrofuran (1:1) with 0.1% BHT and filtered through 0.22 µm PTFE membrane. The extracts were flushed with nitrogen and stored at -80°C prior to HPLC analysis.

Care was taken to evaporate just to dryness, to prevent degradation and preferential adhesion of carotenoids to vial walls (Emenhiser, Englert, Sander, Ludwig, & Schwartz, 1996). Sample preparation and extraction procedures were performed under reduced light, and all glassware and tubes were wrapped in

aluminum foil to avoid contact with light. Extracts were analyzed within three days of extraction, or after one freeze-thaw-cycle (frozen storage at -20 ^{o}C).

2.5. HPLC-PDA analysis

Separation and quantification of carotenoids were carried out on a Waters Acquity^{TM} UPLC-PDA system, using an existing method developed by the Analytical Services unit, School of Agriculture and Food Sciences, The University of Queensland (J. Waanders, personal communication, March 2012). Isocratic elution was performed at 2 mL/min on a Hypersil® OBS C18 (250 x 4.6 mm, 5 um i.d.) RP column (ThermoQuest) using a mobile phase of methanol:tetrahydrofuran:water (67:27:6). The column temperature was maintained at 25°C. An injection volume of 5 µL was used, and UV-Vis spectra of column eluent were recorded from 210-498 nm.

β-carotene was identified by comparing the retention time and UV-Vis absorption spectrum with all-*trans*-β-carotene reference standard (>98% purity, Sigma-Aldrich, NSW, Australia) and xanthophylls were tentatively identified by comparing to those of published literature data. Xanthophylls concentrations were calculated as β-carotene equivalents. β-Carotene calibration curve for quantification was constructed by plotting peak area against concentration (µg) (r^2=0.999). β-Carotene working standards (0.2, 0.5, 1, 5, 10, 25, 50 µg/mL) were prepared fresh on a daily basis from a β-carotene stock (50 µg/mL) in methanol:tetrahydofuran (1:1) with 0.1% BHT, and injected daily. β-Carotene standard concentration was calculated using spectrophotometric absorbance at 453 nm and a molar absorption coefficient of 2592. β-Carotene stock solution was found to be stable for two months at -20°C (<5% loss). A typical HPLC chromatogram of *KP* mango is shown in Fig 2.

2.6. Moisture analysis

Moisture contents of the pureed mango and chewed particles (2-5 g) were determined by vacuum oven drying (65°C for 24 h).

2.7. Confocal laser scanning microscopy (CLSM)

Autofluorescence of carotenoids was detected using CLSM (LSM 700, Carl Zeiss, Germany), differential interference contrast (DIC) and Zen (Black) 2011 software. Observations of carotenoid chromoplasts were carried out under 10x, 20x and 63x objective lens, at an excitation λ of 488 nm, emission λ below 488 nm,

and laser power intensity of 2%. Fluorescence of cell walls was observed at excitation λ of 355 nm and emission λ from 300-440 nm, after staining with Calcofluor.

2.8. Statistical analysis

Significant differences between mean values of carotenoid quantification were tested using one-way ANOVA, while differences between chewers and particle sizes were determined using Tukey's HSD multiple rank test ($p<0.05$) (Minitab v.16, USA).

3. Results and Discussion

3.1. Carotenoid composition and content

A typical HPLC carotenoid profile of *Kensington Pride (KP)* mango contains 12 peaks (Fig 2). Peaks 1-4, 6, 8-10, and 5, 7, 11 were tentatively identified as all-*trans*-violaxanthin and 9-*cis*-violaxanthin, and/or their derivatives respectively. The absorption spectra of all-*trans*-violaxanthin (λ_{max} 416, 441, 472 nm) and 9-*cis*-violaxanthin (λ_{max} 413, 436, 465 nm) are similar to those reported by Ornelas-Paz, Failia, Yahia and Gardea (2008). The 9-*cis*-violaxanthin isomer was distinguished from the all-*trans* isomer based on a characteristic 3 nm hypochromic shift (Ornelas-Paz, Yahia, & Gardea, 2007). The identification of *trans* and *cis*-violaxanthins is based on comparison to published absorption spectra, and has not been characterized as free xanthophylls or xanthophyll esters so in this study, they are collectively termed as xanthophylls. Peak 12 was identified as β-carotene by comparing elution time and spectral maximum (λ_{max} 453, 481 nm) to an authentic standard (λ_{max} 453, 481 nm).

KP mango carotenoid composition is reported for the first time, and is comprised principally of all-*trans*-β-carotene (54%), followed by all-*trans*-violaxanthin (34%) and some 9-*cis*-violaxanthin (12%). Similar compositions have been recorded for *Tommy Atkins, Manila, Ataulfo, Haden* and Taiwanese cultivars (Chen et al., 2004; Manthey & Perkins-Veazie, 2009; Ornelas-Paz et al., 2007), with β-carotene being the predominant carotenoid in mangoes, although β-carotene as a percentage of total carotenoids can vary from 48 to 84% (Godoy & Rodriguez-Amaya, 1989) depending on the cultivar or fruit physiological maturity stage. The β-carotene content in *KP* mango (1282-2081 µg/100 g FW) is higher than in most other cultivars (de la Rosa et al., 2010), where it ranges from 191 to 1340 µg/100 g, with the exception of *Ataulfo* cultivars.

In contrast, all-*trans*-violaxanthin (930-1150 µg/100 g FW) and 9-*cis*-violaxanthin (318-425 µg/100 g FW) was present in lower concentrations to those reported for other cultivars (Mercadante & Rodriguez-Amaya, 1998; Mercadante, Rodriguez-Amaya, & Britton, 1997; Ornelas-Paz, Failla, et al., 2008). β-Carotene shows ~40% variability between individual mangoes, reflecting fruit to fruit variation (Hewavitharana, Tan, Shimada, Shaw, & Flanagan, 2013), which is large even for fruits from the same source. *Keitt* mangoes grown in different regions of Brazil also had a two-fold difference in β-carotene content (Mercadante & Rodriguez-Amaya, 1998), indicating that environmental effects may have a similar influence on carotenoid content as cultivar-related differences.

3.2 Mastication, blending, particle size and carotenoid locations

Carotenoids are observed within globules in the cells of mango flesh (Fig 3 A), supporting similar observations from other chromoplast morphology studies (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012; Vasquez-Caicedo, Heller, Neidhart, & Carle, 2006). During blending to a puree, the high shear rate and sharp blade breaks up both cell walls and globules to an almost homogenous mixture (Fig 3 B). The puree consists of 5-10 µm cell components, with some containing carotenoids as evidenced by their color (Fig 1 B(i)) and autofluorescence (Fig 1 B(ii)); however, most structural cell walls are no longer present.

Mastication confers actions that are not replicable with a cutting blade such as compression, compaction, squashing, and lubrication of food material with saliva to form a cohesive bolus. While these actions collectively encapsulate carotenoids, teeth cutting or slicing can be considered a prerequisite for releasing cell contents, where the physical barriers of plant cell walls are ruptured. In larger chewed fractions (captured on 5.6 and 2.8 mm sieves), clusters of intact and stacked cells encapsulating carotenoids are held tightly together by vascular fibre strands (Fig 4A, 4B). The 1 mm tissue fraction comprises single cells and cell fragments, while a reduction in cell size is observed (Fig 4C). In the 0.075 mm fraction, the cells are more sparsely dispersed with empty pockets of ruptured cells (Fig 4D); additionally, ‘free’ carotenoids are detected, indicating release from the broken mango cells (Fig 4D(ii)).

Hutchings and Lillford (1986) proposed that boluses should reach a degree of structure and lubrication before swallowing, and so information on the bolus water content before and after chewing could give a

measure of saliva incorporated into the masticated matrix. The moisture content of masticated fractions (96±1%, 98±0.2%, 99±0.3% in 2.8, 1, and 0.075 mm fractions respectively) was always higher than in fresh mango (84±1%), showing a considerable portion of saliva is retained by the boluses during chewing.

3.3 Release of carotenoids from solid particles is dominated by small intestinal digestion

Most in vitro digestion studies ignore oral mastication or replace it with mechanical processing, and only a few studies have investigated the effects of mastication on nutrient bioaccessibility (Bornhorst et al., 2014; Epriliati et al., 2009; Hoerudin, 2012; Ranawana, Monro, Mishra, & Henry, 2010). Thus, carotenoid release from solid chewed fractions as a starting material was of particular interest in this study. Relating the release of carotenoids in the fresh mango tissue to that of the expectorated boluses posed an initial challenge due to the constant production and dilution of saliva during chewing. Therefore, the bioaccessibility of each particle size fraction was determined as the bioaccessible fraction in the supernatant relative to the sum of combined contents in the digesta supernatant and bolus pellet following digestion, rather than to the absolute content of fresh mango. As such, the fraction of carotenoids readily lost to the aqueous environment, i.e. the liquid phase in the mouth during chewing and washing during the wet sieving process, is not taken into account, in contrast to the puree, which still contains the liquid phase.

After simulated gastric digestion (1 h), mango cells and vascular fibres were still intact (Fig 5A, 5C), and apparently encapsulating the carotenoids, indicating that acidic hydrolysis did not have a major role in breaking down the cell walls or releasing the carotenoids. This is consistent with a relatively low level of release from chewed bolus particles under these conditions (Fig 6). In contrast, the effect of the emulsifying activity of bile salts on carotenoid aggregation was evident after subsequent small intestinal digestion in vitro. Cellular-trapped and ‘free’ carotenoids are shown as assorted clusters of globular aggregates (Fig 5B, 5D), which are consistent with a significant increase in release of β-carotene from 8% to 33% (Fig 6A), and xanthophylls from 20% to 57% (Fig 6B) in the chewed solid fractions following in vitro small intestinal digestion. This illustrates the critical importance of bile salts for carotenoid release into aqueous digesta. Being lipophilic, carotenoid dissolution in micelles is essential, unlike water-soluble polyphenols that are readily dissolved in aqueous digesta. Other studies of carotenoid release, notably from tomato, have shown that the presence of triglyceride oils increases bioaccessibility (Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012; Huo, Ferruzzi, Schwartz, & Failla, 2007), presumably by facilitating the transfer of

carotenoids to the emulsion phase. However, unlike tomatoes, mango fruit is not typically consumed with oil so this was not investigated in the current study.

The release of β-carotene from mango puree (67%) following small intestinal digestion is substantially greater than from chewed particles (<33%), and likewise for xanthophylls (puree: 75% and chewed particles: <57%). Hedren, Diaz and Svanberg (2002) confirmed that mechanical homogenization leading to carrot cell rupture, increased β-carotene release from 3% to 21% with an expansion of surface area. In addition, Reboul et al. (2006) reported that for carrots, juicing increased bioaccessibility levels from 3% to 14%. However, the absolute values of released carotenoids cannot be directly compared between puree and chewed particle forms as the latter is expected to have lost readily released carotenoids during the chewing and sieving/washing stages. Due to the relatively low % release following in vitro gastric treatment, particularly for β-carotene, we propose that the difference in carotenoid release between puree and chewed particles, after in vitro gastric digestion, provides an estimate of the fraction of carotenoids lost during the oral processing and isolation of solid chewed particles, as indicated in Fig 6 A.

A greater relative % release is observed for the xanthophylls in comparison to β-carotene, presumably due to xanthophylls being less hydrophobic. Transfer efficiency seems to be influenced by solubility as reflected in the micellarised localisation of different carotenoid types. Carotenes are embedded in the triacylglycerol-rich core of micelles, while xanthophylls with more hydroxyl or other functional groups are more polar (Matsuno et al., 1986; Tyssandier et al., 2003), and are expected to reside closer to the surface monolayer, together with proteins, phospholipids, and partially ionised fatty acids (Canene-Adams & Erdman, 2009). This suggests that xanthophylls are more readily incorporated into lipid-bile micelles (Garrett, Failla, & Sarama, 1999; Van Buggenhout et al., 2010), although this may vary amongst green vegetables containing membrane or protein-bound chloroplasts (Failla, Huo, & Thakkar, 2008).

Further to this, there is a fraction of carotenoids that is not released following small intestinal digestion (Fig 6). There is still a limited extent of bioaccessibility from the solid chewed particles (20-30% for β-carotene and 40-50% for xanthophylls) or incomplete bioaccessibility after pureeing (65-75%). We propose that the incomplete bioaccessibility of purees is due to the crystallinity of mango carotenoids or residual chromolast structure, since there is no evidence for the presence of intact cell wall material. For the chewed particles,

additional restrictions on bioaccessibility are proposed to arise from residual embedding in cell wall residues (Fig 5D). Pectin from fruit matrices has been suggested to interfere with micelle formation by partitioning bile salts and fat in the pectin gel phase (Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011; Parada & Aguilera, 2007; Rock & Swendseid, 1992) that is necessary for the absorption of lipophilic carotenoids.

3.4 Higher carotenoid concentration in particles from coarse chewing and in larger particle size fractions

Larger absolute amounts of β-carotene and xanthophylls were found in equivalent sieve fractions from a coarse chewer compared with a fine chewer ($p<0.05$) (Table 1), for both the bioaccessible carotenoids and those trapped in the residual plant material following in vitro small intestinal digestion. This relatively large difference (coarse chewed particles typically have about 30% higher carotenoid concentration than fine chewed particles – Table 1) suggests that fine chewing causes more carotenoids to be released into the solution phase, which in this experiment either remained in the mouth or were removed during sieve capture of particles. In contrast, the percentage release of either β-carotene or xanthophylls from all particle sizes was very similar for both coarse and fine chewers (Fig 6), apart from xanthophylls from the smallest fraction. This illustrates that, in this trial, the type of chewing (coarse vs fine) had a larger impact than the chewed particle size in determining total release of carotenoids from mango fruit.

In addition, total carotenoids were present in higher concentrations in larger particles, which suggest a distribution effect due to a greater mass of plant cell walls, cell clusters and insoluble fibre network in larger particles that entraps more carotenoids. The highest concentration per 100 g (fresh weight) was found in the 2.8 mm fraction, consisting of bulky cell clusters, followed by the 1 mm fraction containing single cells and some cell fragments, while the lowest concentration was found in the 0.075 mm fraction, which consists mostly of fragmented cells.

The smallest particle size (< 500 µm, > 75 µm) fraction resembles a fine pulp or ‘mash’ (Fig 1A/B(iv)), for which the increased surface area explains the higher % release in the smaller particles (Fig 6), in agreement with Lemmens et al. (2010) and Hedren et al. (2002), who reported that smaller particles had a higher release or digestibility. Netzel et al. (2011) also showed that disruption of the cell wall matrix led to improved release rates, with bioaccessibility of carotenoids in single carrot cells (70-80 µm) increasing two-fold compared to 230 µm cell clusters. There is a general trend for smaller particles to be associated with a

greater % release of carotenoids, but the differences between particle sizes or fine vs coarse chewing (Fig 6) are unexpectedly small. Whilst there is indeed a (small) particle size effect on bioaccessibility, this may be secondary to the extent of chewing for soft tissues such as mango, which contrasts with carrot tissues where a single cell wall appears to be enough to retain carotenoids (Tydeman et al., 2010a). This contrast with carrots is likely to be a consequence of the robustness of the cell wall matrix. In carrots and many other vegetables, cell walls are relatively robust and can survive cooking processes. In ripe mango and possibly other ripe fruits, chewing and *in vitro* digestion results in a gel-like solid structure (Fig 5) without a discrete wall. Therefore, phytonutrient bioaccessibility may vary amongst fruits, vegetables, grains and legumes due to differences in cell wall thickness and structure, which determine how intact cellular structures remain after chewing and digestion.

4. Conclusions

This study of mastication effects on carotenoid release emphasises the importance of including chewing in *in vitro* digestion studies. Mastication confers a combination of size reduction and compaction processes as part of the digestive process, with plant cell wall structures being one of the limiting factors for carotenoid release, and chromoplast location of carotenoids being another. Some carotenoids (25-33%) may be present as crystallites or may remain trapped in the residual plant matrix, and could potentially be fermented in the large intestine, releasing more carotenoids during degradation of cell walls there. This warrants further investigation of carotenoid release following colonic fermentation of *in vitro* digestion residues. Particle size and the type of chewing resulted in differences in the relative amounts of carotenoids in the solid fraction after chewing, but had a surprisingly small impact on carotenoid bioaccessibility from the solid particles, suggesting that cell wall factors are not necessarily the most important in determining carotenoid bioaccessibility in soft tissues in contrast to tissues with more robust cell walls such as carrot.

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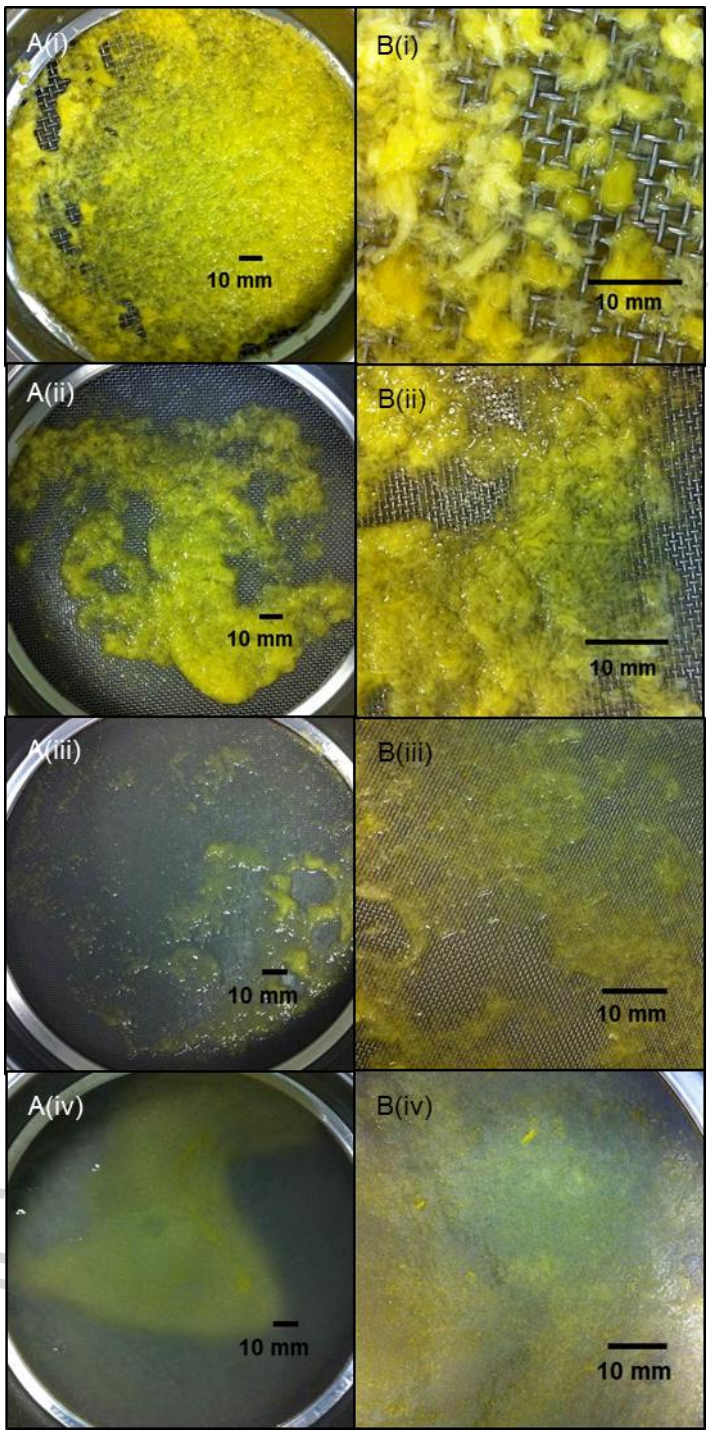

Figure 1. Images of A(i-iv) fractionated and chewed mango boluses particles captured on sieves of screen size 2.8, 1, 0.5 and 0.075 mm respectively, and B(i-v) magnified view of each fraction. Larger particle clumps and vascular fibres are observed in 2.8 mm screen (B(i)) fractions. A finer texture is observed with each decreasing sieve size.

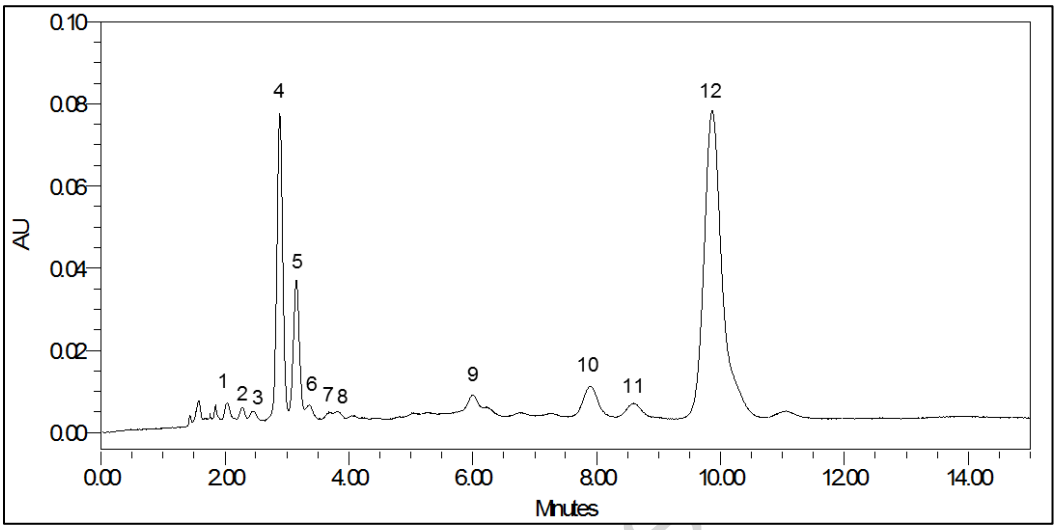

Figure 2. Representative HPLC chromatogram of ripe *KP* mango extract with detection at 453 nm. Peaks 1-4, 6, 8-10 were assigned to all-*trans*-violaxanthin (and derivatives), and peaks 5, 7, 11 were assigned to 9-*cis*-violaxanthin (and derivatives). Peak 12 was identified as all-*trans*-β-carotene.

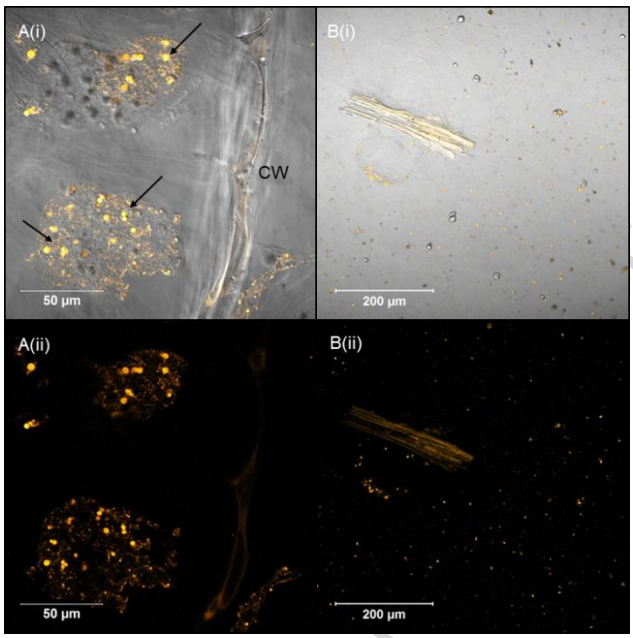

Figure 3. Brightfield (i) and CLSM (ii) images from mango flesh (A) and puree (B) showing orange colour (i) and autofluorescence (ii) of carotenoids. Arrows in A(i) highlight carotenoid-containing chromoplasts, and 'CW' represents cell walls.

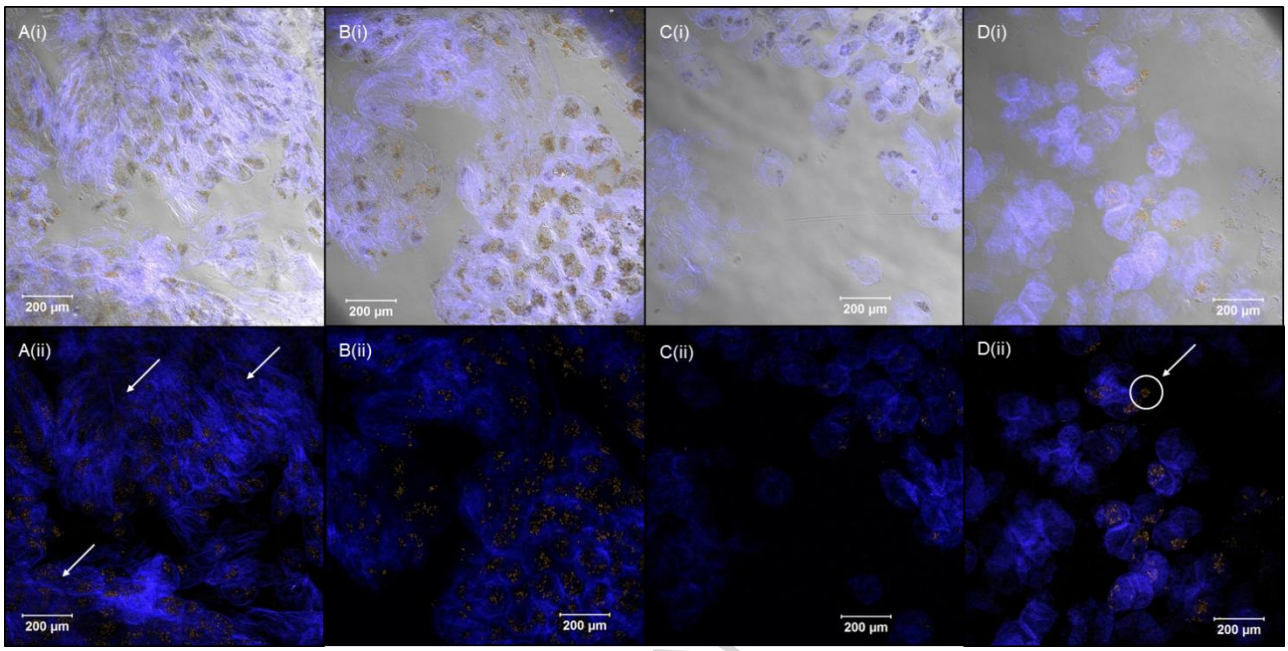

Figure 4. Effect of *in vivo* chewing on mango cellular microstructure for particles captured on sieves of screen size: (A) 5.6 mm, (B) 2.8 mm, (C), 1 mm, and (D) 0.075 mm. A(i)-D(i) differential interference contrast images showing carotenoids (orange) located within residual cellular structures; A(ii)-D(ii) the same fields of view showing fluorescence of carotenoids (orange globules) and cell walls (purple-blue). Arrows in A(ii) show connective vascular fibres and in D(ii) shows released carotenoids.

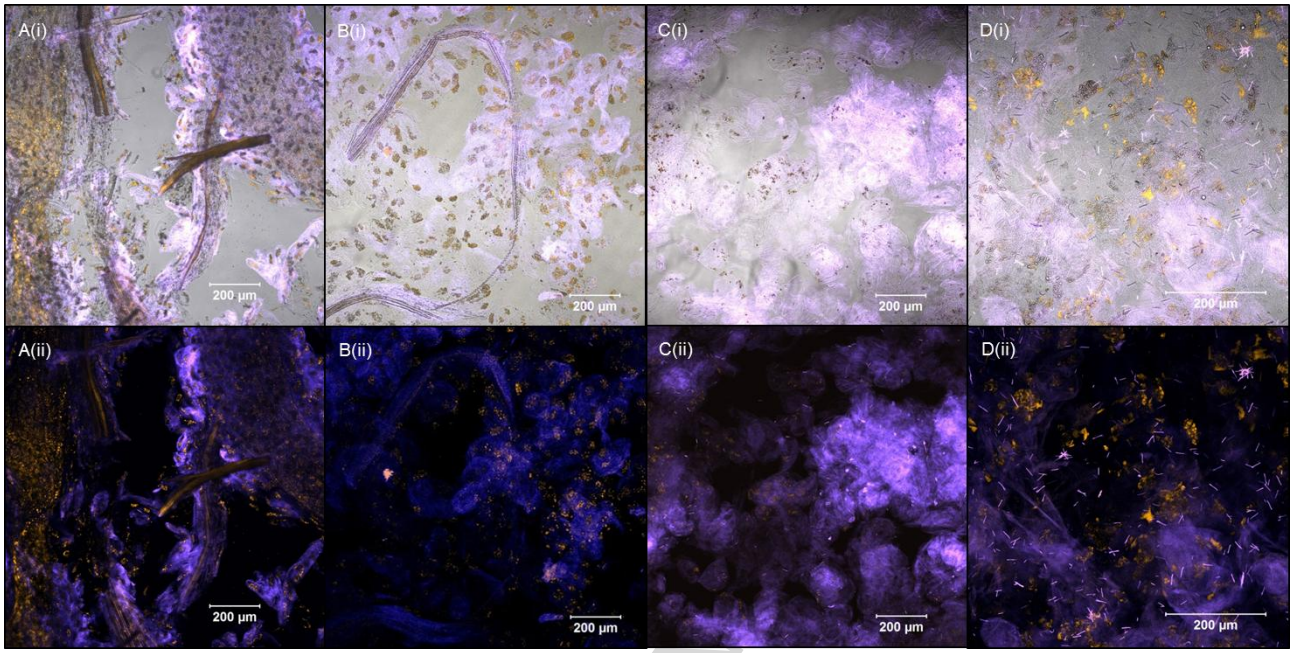

Figure 5. Effect of *in vitro* digestion on two sieve size fractions of chewed mango following *in vitro* gastric digestion: (A) 5.6 mm, (C) 0.075 mm, and following small intestinal digestion: (B) 5.6 mm, (D) 0.075 mm; A(i) – D(i) differential interference contrast; A(ii)-D(ii) fluorescence of carotenoids (orange globules) and cell walls (purple-blue).

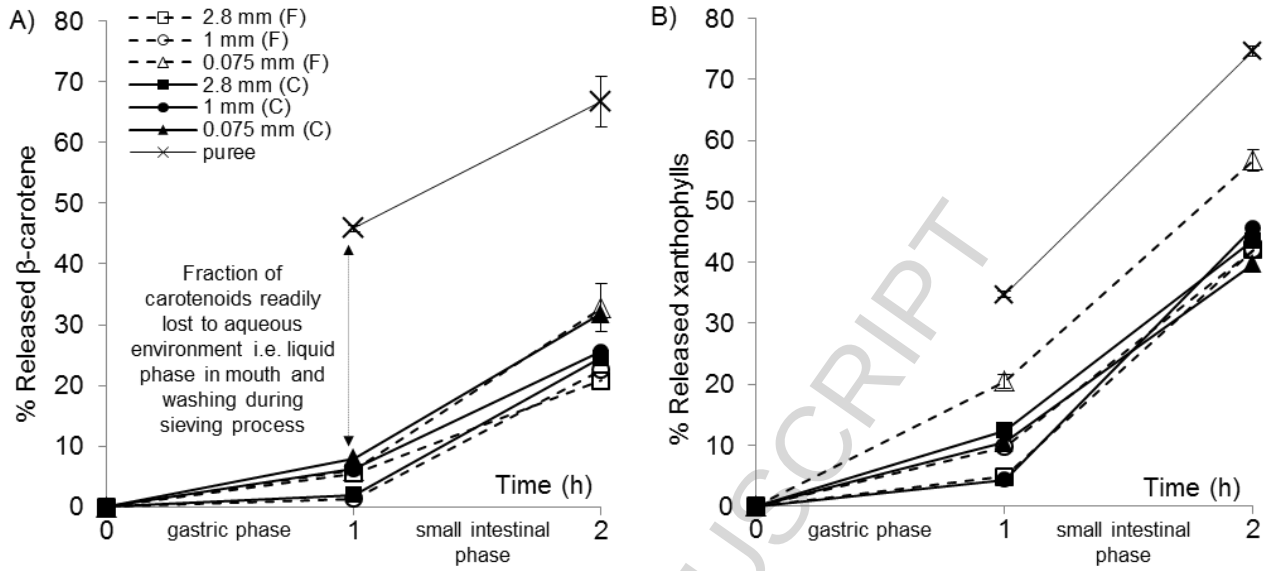

Figure 6. Percentage release of A) β-carotene and B) sum of total xanthophylls and derivatives, from masticated mango fractions and puree following simulated gastric (1 h) and small intestinal (2 h) digestion, into the aqueous digesta phase. Solid particles captured on three sieves (2.8, 1, 0.075 mm) from a fine (F) and a coarse (C) chewer were studied. For clarity, error bars are only shown for puree and 0.075 mm (F) samples.

Table 1. Carotenoid concentrations (µg/100 g FW) in each fraction following digestion *in vitro*

Chewed mango fractions	Gastric digestion	Bioaccessible fraction: Small intestinal digestion	Bioaccessible fraction: β-carotene/ xanthophylls[1]	Trapped in plant matrix: Small intestinal digestion	Trapped in plant matrix: β-carotene / xanthophylls
Coarse					
2.8 mm	48 ± 27^{a}	320 ± 96^{a}	211 ± 90^{a}/127 ± 9^{x}	788 ± 220^{a}	649 ± 198^{a}/159 ± 28^{x}
1 mm	47 ± 12^{a}	249 ± 28^{ab}	154 ± 13^{ab}/115 ± 16^{xy}	561 ± 171^{abc}	447 ± 139^{abc}/132 ± 35^{xy}
0.075 mm	50 ± 23^{a}	199 ± 42^{ab}	152 ± 42^{ab}/93 ± 20^{yz}	397 ± 81^{bc}	326 ± 58^{bc}/ 90 ± 24^{yz}
Fine					
2.8 mm	44 ± 13^{a}	211 ± 14^{ab}	131 ± 14^{ab}/99 ± 5^{xyz}	610 ± 108^{ab}	499 ± 79^{ab}/129 ± 29^{xy}
1 mm	24 ± 19^{a}	192 ± 18^{b}	118 ± 22^{ab}/93 ± 3^{yz}	506 ± 66^{abc}	404 ± 67^{abc}/121 ± 2^{xy}
0.075 mm	34 ± 17^{a}	136 ± 18^{b}	87 ± 20^{b}/68 ± 4^{z}	216 ± 20^{c}	179 ± 15^{bc}/56 ± 10^{z}

Values are means±SD of three independent chewing and digestion experiments. Values with different letters within each column for each chewer denote significant difference ($p<0.05$) in carotenoid concentration.

[1]Xanthophylls consist of violaxanthins and derivatives.

Highlights

- Effect of particle sizes after chewing on mango fruit carotenoid bioaccessibility studied.
- Carotenoids are retained within mango cellular structures after chewing.
- Larger particles contain more carotenoids after chewing and digestion
- Similar β-carotene and xanthophylls % release for each chewed particle size fraction.